Theor Appl Genet (2005) 112: 158–166
DOI 10.1007/s00122-005-0118-9

ORIGINAL PAPER

Hong-wei Cai · Maiko Inoue · Nana Yuyama
Wataru Takahashi · Mariko Hirata · Tohru Sasaki

Isolation, characterization and mapping of simple sequence repeat markers in zoysiagrass (*Zoysia* spp.)

Received: 6 June 2005 / Accepted: 15 September 2005 / Published online: 19 October 2005

Abstract The genus *Zoysia* consists of 16 species that are naturally distributed on sea coasts and grasslands around the Pacific. Of these, *Zoysia japonica*, *Zoysia matrella*, and *Zoysia tenuifolia* are grown extensively as turfgrasses, and *Z. japonica* is also used as forage grass in Japan and other countries in East Asia. To develop simple sequence repeat (SSR) markers for zoysiagrass (*Zoysia* spp.), we used four SSR-enriched genomic libraries to isolate 1,163 unique SSR clones. All four libraries contained a high percentage of perfect clones, ranging from 67.1 to 96.0%, and compound clones occurred with higher frequencies in libraries A (28.6%) and D (11.6%). From these clones, we developed 1,044 SSR markers when we tested all 1,163 SSR primer pairs. Using all 1,044 SSR markers, we tested one screening panel consisting of eight *Zoysia* clones for testing PCR amplifications, from which five unrelated clones, among the eight, were used for polymorphism assessment, and found that the polymorphic information content ranged from 0 (monomorphic loci) to 0.88. Of the 1,044 SSR markers, 170 were segregated in our mapping population and we mapped 161 on existing amplified fragment length polymorphism-based linkage groups, using this mapping population. These SSR markers will provide an ideal marker system to assist with gene targeting, quantitative trait locus mapping, variety or species identification, and marker-assisted selection in *Zoysia* species.

Electronic supplementary material Supplementary material is available in the online version of this article athttp://dx.doi.org/10.1007/s00122-005-0118-9 and is accessible for authorized users.

Communicated by P. Langridge

H. Cai (✉) · M. Inoue · N. Yuyama · W. Takahashi
M. Hirata · T. Sasaki
Japan Grassland Agriculture and Forage Seed Association,
Forage Crop Research Institute, 388-5 Higashiakada,
Nasushiobara, Tochigi, 329-2742 Japan
E-mail: hcai@jfsass.or.jp
Tel.: +81-287-376755
Fax: +81-287-37-6757

Key words SSR-enriched library · Molecular markers · Polymorphism · Genomic constitution · Zoysia spp.

Introduction

The genus *Zoysia* consists of 16 species that are naturally distributed on sea coasts and grasslands around the Pacific. Five species have been identified from southern Hokkaido to the southwest islands in Japan (Kitamura 1989). Of these, *Zoysia japonica* Steud., *Zoysia matrella* Merr., and *Zoysia tenuifolia* Willd. are utilized extensively as turfgrasses, and *Z. japonica* is also used as forage grass in Japan and other countries in East Asia (Shoji 1983; Fukuoka 1989). Zoysiagrass has a chromosome number of 40 and is mostly a cross-pollinated, tetraploid species with a small genome size (421 Mb for *Z. japonica*) (Forbes 1952; Arumuganathan et al. 1999). Two molecular linkage maps have been constructed from interspecific hybrids of *Z. japonica* and *Z. matrella* (Ebina et al. 1999; Yaneshita et al. 1999) on the basis of restriction fragment length polymorphism (RFLP) and amplified fragment length polymorphism (AFLP) markers, and we have reported an AFLP-based high-density linkage map of *Z. japonica* (Cai et al. 2004). However, RFLP analysis requires a large amount of genomic DNA and is time-consuming and costly, and AFLP markers are dominant and AFLP-based map information is difficult to transfer to a different mapping population. Therefore, more easily used PCR-based, co-dominant markers will be needed for the *Zoysia* species.

The genomes of all eukaryotes contain a class of sequences termed simple sequence repeats (SSRs) (Tautz et al. 1986) or microsatellites (Litt and Luty 1989). SSRs, with tandem repeats of a basic motif of ≤ 6 bp, have emerged as important sources of ubiquitous genetic markers for many eukaryotic genomes (Wang et al. 1994). Although the development of SSR markers takes time and cost, unlike other markers, including RFLP, random amplified polymorphic DNA (RAPD) and AFLP markers, SSR markers have the advantages of

being PCR-based, multi-allelic, co-dominant, and highly polymorphic. Therefore, SSRs have been used in many studies, such as linkage map construction (Röder et al. 1998), gene tagging (Peng et al. 1999; Xie et al. 2002), genetic diversity (Fahima et al. 2002; Huang et al. 2002), and evolutionary studies (Matsuoka et al. 2002). SSR markers have been developed in many plant species, including most major crops such as rice (Wu and Tanksley 1993; Akagi et al. 1996; Panaud et al. 1996; Chen et al. 1997, 2002; Temnykh et al. 2000, 2001; McCouch et al. 2002), maize (Taramino and Tingey 1996; Sharopova et al. 2002), sorghum (Bhattramakki et al. 2000; Kong et al. 2000; Schloss et al. 2002), wheat (Röder et al. 1995, 1998; Gao et al. 2003), barley (Ramsay et al. 2000; Kantety et al. 2002), and other important economic plant species (Jarret et al. 1997; Cordeiro et al. 2000; Danin-Poleg et al. 2001; Tang et al. 2002). Recently, SSR markers have been developed in forage crops such as perennial ryegrass (Jones et al. 2001), Italian ryegrass (Hirata et al. 2000), white clover (Kolliker et al. 2001; Barrett et al. 2004), timothy (Cai et al. 2003), and tall fescue (Saha et al. 2004, 2005). A small number of SSR markers in zoysiagrass have been reported (Tsuruta et al. 2003).

Here, we report the isolation, characterization, and development of 1,044 *Zoysia* SSR markers from four SSR-enriched genomic libraries. One screening panel, consisting of eight *Zoysia* clones, was tested with all 1,044 SSR markers to detect polymorphism levels and identify a set of loci suitable for framework mapping. We also mapped 170 SSR markers on previously constructed AFLP map.

Materials and methods

Plant materials

A *Zoysia* clone, F08, which is an F_1 plant selected from a cross between a *Z. japonica* clone and a *Z. matrella* clone, was used to construct the SSR-enriched genomic library because we wanted to develop SSR markers that could be used for both *Z. japonica* and *Z. matrella*. A panel of *Zoysia* clones, native to Japan, was used for testing PCR amplification and five of them were used for polymorphism assessment. These clones were Tawarayama Kita 1 (from Kumamoto, located at South part of Japan), Syuri 1 (from Okinawa, located at South island of Japan), F02, Muroran 2 (from Hokkaido, located at North part of Japan), Matsushima 5 (from Kumamoto), F08, A29 (from Iwate, located at North part of Japan), and Matsushima 2 (from Kumamoto). All eight clones came from the collection of the Japan Grassland Agriculture and Forage Seed Association at the Forage Crop Research Institute. Among them, F02 is a selfed progenitor of Tawarayama Kita 1, F08 is the F_1 of Muroran 2 and Matsushima 5, and, according to our results of SSR analysis, Matsushima 2 is most probably same as Matsushima 5, which was collected from the same site. Based on the judgment of morphological characters, Matsushima 5 and Matsushima 2 were classified as *Z. matrella*, while the other five clones, except F08, are *Z. japonica*.

A total of 78 individuals were randomly selected among the selfed progeny of clone F02 to establish a mapping population. F02 was initially considered to be a clone selected from the progeny of a cross between ecotypes 'Tawarayama Kita 1' and 'Syuri 1.' Our AFLP data, however, suggest that this clone is most probably a selfed progeny of the female parent, 'Tawarayama Kita 1,' (Cai et al. 2004).

Genomic DNA was extracted from young leaves by the CTAB method (Murray and Thompson 1980).

Construction and sequence analysis of SSR-enriched genomic libraries

Four SSR-enriched genomic libraries (CA, GA, AAG, and AAT) were constructed by the Genetic Identification Services (GIS, Chatsworth, CA, USA) from the *Zoysia* clone F08, and the sequences (about 700 bp long) were obtained at Dragon Genomics (Yokkaichi, Mie, Japan) (details as described in Cai et al. 2003). After sequencing, the Phred quality scores (logarithmically linked to error probabilities, ranging from 4 to about 60) (Brent et al. 1998) were calculated using software Phred (Codon Code Inc., Version 0.000925c), and clones with a Phred quality score >15 and longer than 100 bp were used for primer design.

Sequence checking and primer design

Sequences containing at least five di-, tri-, tetra-, or penta-nucleotide repeats were selected. The SSR structure was defined in terms of four categories, according to Jones et al. (2001): perfect repeats of the form $(N_1N_2)x$ or $(N_1N_2N_3)x$; imperfect repeats of the form (e.g., $N_1N_2N_1N_2N_2N_2N_1N_2N_2N_1N_2N_2N_2N_1N_2$,...,etc.); interrupted repeats of the form $(N_1N_2)x(N)y(N_1N_2)z$ or $(N_1N_2N_3)x(N)y$ $(N_1N_2N_3)z$; and compound repeats of the form $(N_1N_2)x(N_3N_4)y$, $(N_1N_2N_3)x(N_4N_5N_6)y$, or $(N_1N_2)x(N_3N_4N_5)y$.

All sequences containing SSRs were checked to identify duplicates, using Sequencher 4.02 software (Gene Codes Corporation, Ann Arbor, MI, USA). Only unique SSR clones, with sufficiently long flanking sequences (>15 bp) to generate both forward and reverse primers, were used for primer design. Primer pairs (mostly with length of 15–25 bp), flanking the SSR motif, were designed with the program Primer 0.5 (Lander, Cambridge, MA, USA). The annealing temperature was set at 55°C.

Database searches

All unique SSR sequences were searched against the nucleotide databases, using BLAST algorithms (Altschul

et al. 1997). The BLAST network service provided at the DDBJ was used to identify the relationships between the SSR sequences for known genes. BLASTX searches were run on 28 August 2005, and a bits score >80 and E-value $\leq 1\times10^{-10}$ was considered to be significant.

Polymerase chain reaction and fragment analysis

An M13-tagged forward primer (Rampling et al. 2001) method was used in the PCR reaction. The primers used were 5 pmol labeled M13 (−29) primers (IRD700- or IRD800-CACGACGTTGTAAAACGAC; Li-COR, Lincoln, Nebraska, USA), 1 pmol 5′-tagged forward primer, and 5 pmol reverse primer. The 5′-tagged forward primer, for each particular SSR, had the M13 sequence added to its 5′ end.

SSRs were amplified under the following PCR conditions: a 'touchdown' PCR consisting of 94°C for 5 min; two cycles of 94°C for 1 min, 65°C for 1 min, and 72°C for 1.5 min; 10 cycles of 94°C for 1 min, 65–55°C for 1 min decreasing by 1°C/cycle, and 72°C for 1.5 min; 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1.5 min; followed by 72°C for 7 min and 4°C as the holding step.

To detect SSR markers, the PCR products were analyzed in 6% denatured acrylamide gel with a LI-COR sequencer (LI-COR, Lincoln, Nebraska, USA).

Primer evaluation

All primer pairs were screened on the panel of eight *Zoysia* clones for their ability to yield an amplification product of the expected size and to detect polymorphisms. We calculated the number of alleles and polymorphic information content (PIC; Hedrick 1985; Saal and Wricke 1999, Jones et al. 2001) of primers that detected polymorphisms. The formula of PIC is $\mathrm{PIC} = H = 1-\sum p_i^2$, where p_i is the frequency of the i-th allele out of the total number of alleles in the sample. Five unrelated clones – Tawarayama Kita 1, Syuri 1, Muroran 2, Matsushima 5, and A29 – were used for PIC calculation.

Data scoring and linkage analysis

Since the mapping population was derived from the selfed progeny of one clone, only two types of data were possible (only one band appeared in the parent clone and segregated as 3:1 in the population or two bands appeared in the parent clone and segregated as 1:2:1 in the population). Most SSR markers were co-dominant markers, segregating as 1:2:1, but the linkage phase was not known for either type of data. Therefore, the mapping data were scored as cross-pollination data, according to the definition of JoinMap 3.0 (Stam 1993). The linkage map construction was performed with JoinMap 3.0 under the cross-pollination algorism, which can auto-detect the linkage phase from the marker data. The parameters of LOD = 5.0 and a maximum distance of 30 cM were used to group linked markers and the Kosambi mapping function was used (Kosambi 1944). The linkage map was drawn using MapChart 2.0 (Voorrips 2002).

Results and discussion

SSR isolation

We sequenced 4,000 clones (1,000 from each of four libraries). Of these, 3,268 clones (81.7%) contained SSR sequences showing a high level of redundancy (2,500 clones, 76.5% of SSR-containing clones). After we had excluded a few clones whose sequences flanking the SSR motifs were too short to allow us to design both forward and reverse primers, we identified 768 unique SSR clones (23.5% of SSR-containing clones) that we used to design primers (Table 1). The percentage SSR marker isolation efficacy (23.5% of SSR-containing clones) was lower than the results obtained in sunflower (Tang et al. 2002) and Italian ryegrass (Hirata et al. 2000), through the use of SSR-enriched libraries produced by GIS; perhaps because our libraries have a very high level of redundancy.

Our level of redundancy was very high—(76.5%)-higher than that in other species (24% in *Melaleuca alternifolia*, Rossetto et al. 1999; 16% in *Lolium perenne*, Jones et al. 2001; <1% in sugarcane, Cordeiro et al. 2000; 40.8% in timothy, Cai et al. 2003). Redundancy was mostly found within libraries. This redundancy was most probably caused by clone duplication, especially in libraries A, C, D.

The percentage of unique SSR clones in library B (motif GA/TC, 49.9%) was higher than in the others (12.2–16.0%); this result agreed with that found in timothy (Cai et al. 2003). On the basis of the above results, we sequenced another 2,000 clones (called B2) from library B and isolated 395 more unique SSR clones, which were then used to design primers.

Over 1,163 unique SSR sequences were searched against the nucleotide databases of DDBJ, and a total of 108 sequences showed significant homology with sequences of other plant species; of them, most showed significant homology with rice genomic or cDNA sequences with unknown functions, but 26 showed significant homology with genes (Table 2), and no sequences showed significant homology with transposable elements.

Characterization of SSR loci

SSR loci were classified by repeat type and structure (Table 1). All four libraries contained perfect clones at

Table 1 Efficacy of SSR isolation, frequency of repeat types, and working primers from four *Zoysia* SSR libraries

Library	Motif	SSR clones	Unique SSR clones	Repeat type				Primer evaluation			
				Perfect	Compound	Imperfect	Interrupted	Polymorphic	Monomorphic	Multiple band	No amplification
A	CA/TG	873 (87.3%)	140 (16.0%)[a]	94 (67.1%)	40 (28.6%)	1 (0.7%)	5 (3.6%)	125 (89.3%)	7 (5.0%)	1 (0.7%)	7 (5.0%)
B	GA/TC	818 (81.8%)	408 (49.9%)	371 (90.9%)	23 (5.6%)	1 (0.2%)	13 (3.2%)	380 (93.1%)	3 (0.7%)	2 (0.5%)	23 (5.6%)
C	AAG/TTC	801(80.1%)	125 (15.6%)	120 (96.0%)	5 (4.0%)	0 (0.0%)	0 (0.0%)	82 (65.6%)	20 (16.0%)	18 (14.4%)	5 (4.0%)
D	AAT/TTA	776 (77.6%)	95 (12.2%)	73 (76.8%)	11 (11.6%)	7 (7.4%)	4 (4.2%)	78 (82.1%)	3 (3.2%)	10 (10.5%)	4 (4.2%)
B2	CA/TG	–	395	357 (90.4%)	22 (5.6%)	1 (0.3%)	15 (3.8%)	338 (85.6%)	8 (2.0%)	4 (1.0%)	45 (11.4%)
Total		3,268 (81.7%)[b]	1,163	1,015 (87.3%)	101 (8.7%)	10 (0.9%)	37 (3.2%)	1,003 (86.2%)	41 (3.5%)	35 (3.0%)	84 (7.2%)

[a] % of SSR-containing clones
[b] Except B2

very high frequencies, with an average of 87.3% (ranging from 67.1 to 96.0%). Compound repeats were contained at higher frequencies in libraries A (28.6%) and D (11.6%) than in libraries B (5.6%), and C (4.0%). Imperfect clones were contained mostly in library D (7.4%).

Table 3 shows the proportions of different repeat motifs in each of the structural categories, by library. In all four libraries, the predominant motif was the expected type, e.g., CA/TG for A, GA/TC for B, AAG/TTC for C, and AAT/TTA for D. In the most frequent dinucleotide- and trinucleotide-motif-containing perfect clones, the average repeat numbers were 16.3 (CA/TG), 18.7 (GA/TC), 10.1 (AAG/TTC), and 11.5 (AAT/TTA). The average repeat numbers in compound clones were 28.9 (15–47) in library A and 29.1 (9–45) in library B (average of B and B2) (data not shown).

As pointed out by Cardle et al. (2000), the most common dinucleotide motif found in plant genomic sequences is AT/TA, followed by GA/CT and CA/GT, and the most common trinucleotide motifs are AAT/TAA and ATC/TAG. Although AAG/TTC dominates in *Arabidopsis thaliana*. Although AT/TA is the most common dinucleotide motif in plant genomic sequences, this motif is not usually used in SSR-enrichment procedures, owing to its self-complementary nature; therefore, we did not use AT/TA in our study. Of 658 perfect SSRs (except B2), the motifs occurring at the highest rates were GA/TC (57.3%), CA/GT (14.3%), AAG/TTC (16.9%), and AAT/TAA (10.3%). These results are close to those reported by Cardle et al. (2000) and Cai et al. (2003).

Primer evaluation

Of the 1,163 primer pairs tested, 1,003 (86.2%) could amplify polymorphic products (example shown in Fig. 1); in the eight *Zoysia* clones used, with product

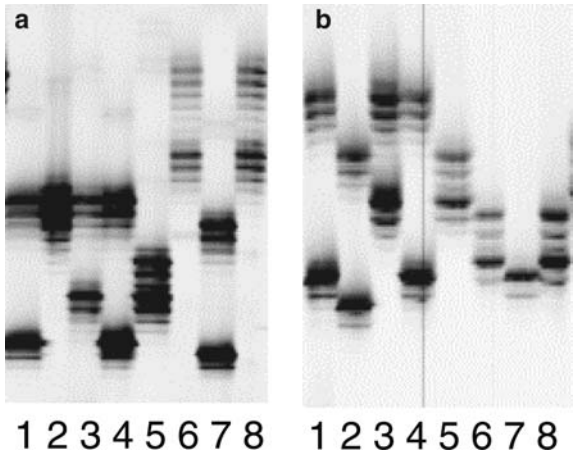

Fig. 1 PCR products amplified by the loci *ZB02N16* (**a**) and *ZB03B02* (**b**) in eight clones of zoysiagrass. *Lane 1* Matsushima 2; *lane 2* A29; *lane 3* F08; *lane 4* Matsushima 5; *lane 5* Muroran 2; *lane 6* F02; *lane 7* Syuri 1; *lane 8* Tawarayama Kita 1

Table 2 High-scoring matches against the DDBJ databases by BLAST search

Clone	Matched clone	Putative identification	Bits score	*E* value
ZB01N19	AY224508	*Oryza sativa* (japonica cultivar-group) isolate 3,1185 putative zinc finger protein mRNA, partial cds	121	2.00E−24
ZB02G20	AB029510	*O. sativa* mRNA for small GTP-binding protein OsRac3, complete cds	206	4.00E−50
ZB02H22	AY163379	*Zea mays* putative ROP family GTPase ROP2 gene, complete cds	196	5.00E−47
ZB02K07	AY581830	*Cynodon dactylon* putative Rieske Fe-S precursor protein mRNA, partial cds	103	5.00E−19
ZB03B03	AF466286	*O. sativa* putative G-box binding protein mRNA, complete cds	103	5.00E−19
ZB03B24	AF112149	*Z. mays* MADS box protein 2 (mads2) mRNA, complete cds	145	1.00E−31
ZB03E06	AF033263	*Z. mays* nonphototropic hypocotyl 1 (nph1) mRNA, complete cds	103	3.00E−19
ZB03G12	AY946350	*O. sativa* microRNA miR396d precursor, gene, partial sequence	109	7.00E−21
ZB03I20	AF244685	*Z. mays* glutathione S-transferase GST 20 mRNA, complete cds	121	2.00E−24
ZB03L04	AY163379	*Z. mays* putative ROP family GTPase ROP2 gene, complete cds	182	6.00E−43
ZB03M23	AY359572	*Z. mays* B73 acc oxidase (ACO15) gene, complete cds	125	1.00E−25
ZB04C05	AY220669	*O. sativa* box C/D snoRNA J37.1, complete sequence	103	5.00E−19
ZB04N13	AJ440217	*O. sativa* a6 gene for plasma membrane H + -ATPase	521	1.00E−145
ZB05A15	AJ938073	*O. sativa* (indica cultivar-group) mRNA for Type A response regulator 4 (rr4 gene)	115	1.00E−22
ZB06A14	AY312574	*Deschampsia antarctica* Rubisco activase beta form precursor (RCA2) mRNA, complete cds; nuclear gene for chloroplast product	107	2.00E−20
ZB06D06	AY312169	*Z. mays* homeobox transcription factor KNOTTED1 (kn1) gene, complete cds	97	9.00E−17
ZB06F08	AY581830	*C. dactylon* putative Rieske Fe–S precursor protein mRNA, partial cds	186	4.00E−44
ZB08J13	AF435642	*O. sativa* CSLC7 mRNA, partial cds	167	4.00E−38
ZB08K21	AY870607	*O. sativa* (japonica cultivar-group) WRKY23 mRNA, complete cds	176	2.00E−41
ZB08M09	AY180106	*Z. mays* cultivar B73 liguleless2 gene, complet cds	125	9.00E−26
ZB08O01	AF112149	*Z. mays* MADS box protein 2 (mads2) mRNA, complete cds	147	3.00E−32
ZC01C22	AB125306	*O. sativa* (japonica cultivar-group) SAPK5 mRNA for serine/threonine protein kinase SAPK5, complete cds	159	6.00E−36
ZC01G15	AY323481	*O. sativa* (japonica cultivar-group) ribosomal L9-like protein mRNA, complete cds	159	8.00E−36
ZC02L16	L05934	*Z. mays* catalase (Cat3) gene, complete cds	88	2.00E−14
ZT-A124	AF470066	*Sorghum bicolor* P-type R2R3 Myb protein (Myb27) gene, partial cds	115	1.00E−22
ZT-C12	AF332176	*Z. mays* beta-expansin 3 (expB3) mRNA, partial cds	127	2.00E−26

size ranging from 70 to 350 bp (mostly in 100–200 bp), 41 (3.5%) amplified monomorphic products, 35 (3.0%) amplified multiple bands, and 84 (7.2%) amplified no bands (Table 1). The percentage of working primers (polymorphic and monomorphic) in our study was 89.7%, similar to those in ryegrass and sunflower (Hirata et al. 2000; Jones et al. 2001; Tang et al. 2002), but higher than in wheat, barley, and timothy (Röder et al. 1998; Ramsay et al. 2000; Cai et al. 2003). Among the 1,003 working polymorphic primer pairs, 51 (5.1%) amplified products in only *Z. matrella*. Most markers had a large number of alleles, ranging from 3 to 9 (supplementary electronic material Table 4). The PIC values ranged from 0 (monomorphic locus) to 0.88 (as shown in Table 4), and the average of PIC values were increased with an increase in the allele numbers, however, the PIC and the numbers of alleles did not increase with an increase in the repeat lengths.

From the results of the primer test for the eight *Zoysia* clones, it was suggested that F08 is a true hybrid F_1 of Muroran 2 and Matsushima 5, and F02 is a selfed progenitor of Tawarayama Kita 1. In addition, Matsushima 2 is most probably same as Matsushima 5 because the two clones showed the same band pattern in all 1,003 polymorphic SSR markers tested. The above results suggested the usefulness of these markers for germplasm

Table 3 Frequencies of motif types in the perfect SSR isolated from four *Zoysia* SSR-enriched libraries

Motif	Library A	Library B	Library C	Library D	Library B2	Total (%)	Average repeat number (range)
CA/TG	92 (97.9%)	1 (0.3%)	1 (0.8%)		3 (0.8%)	97 (9.6%)	16.3 (15–47)
GA/TC	2 (2.1%)	370 (99.7%)	2 (1.7%)	3 (4.1%)	346 (96.9%)	723 (71.2%)	18.7 (9–45)
AAG/TTC			111 (92.5%)		4 (1.1%)	115 (11.3%)	10.1 (4–94)
AAT/TTA				68 (93.2%)		68 (6.7%)	11.5 (6–23)
TCA					1 (0.3%)	1 (0.1%)	6.0
CAA				1 (1.4%)		1 (0.1%)	4.0
CAT			1 (0.8%)			1 (0.1%)	5.0
AA			2 (1.7%)			2 (0.2%)	21 (18–24)
GAGAA				1 (1.4%)		1 (0.1%)	15.0
CAG					1 (0.3%)	1 (0.1%)	6.0
GGA					1 (0.3%)	1 (0.1%)	8.0
TCC			3 (2.5%)		1 (0.3%)	4 (0.4%)	7.0 (5–13)
Total	94	371	120	73	357	1,015 (100%)	

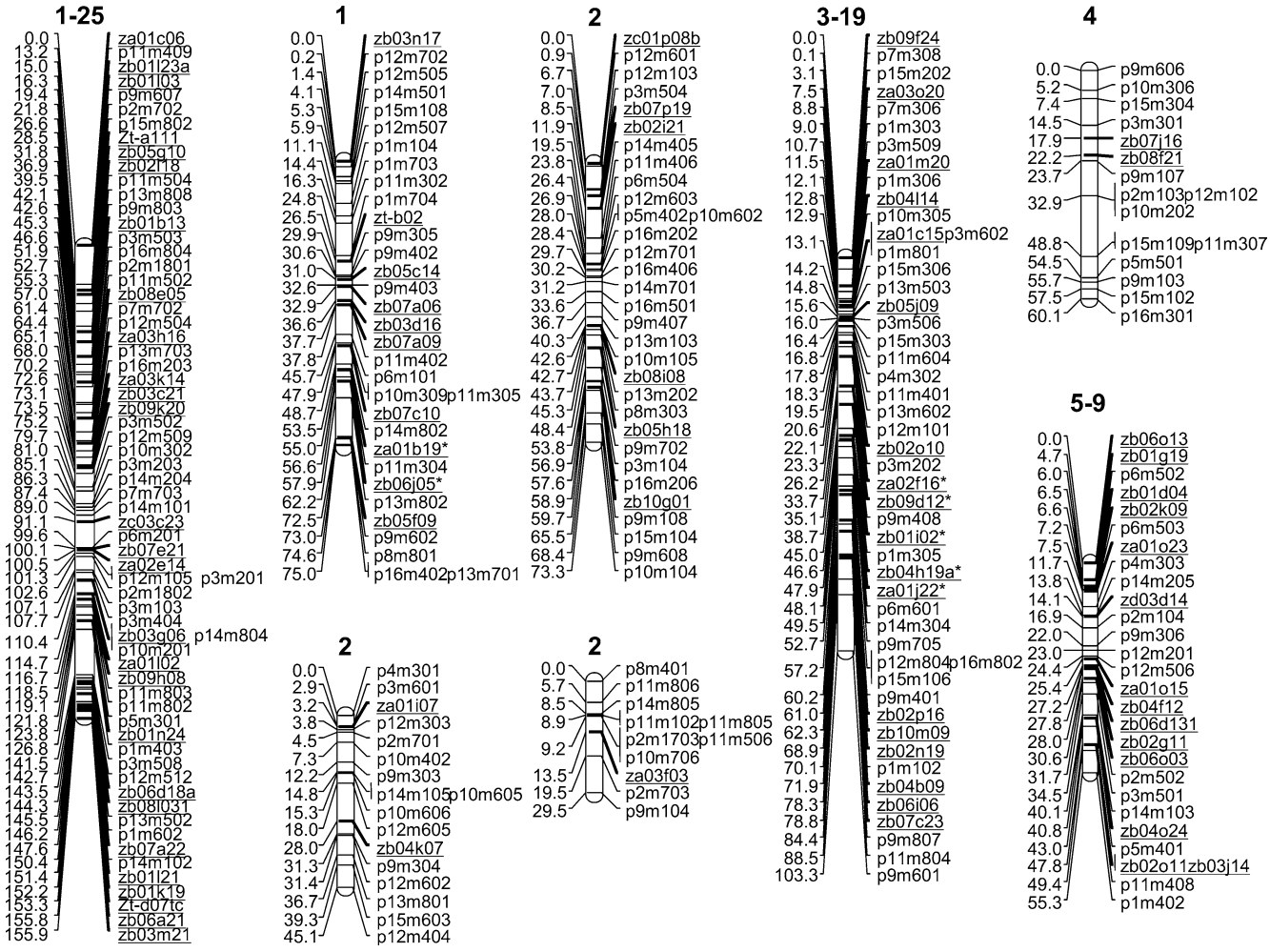

Fig. 2 Linkage map of the *Z. japonica* mapping population based on SSR and AFLP markers. The linkage groups are represented as *vertical bars*, with their numbers (according to Cai et al. 2004) indicated at the *top* and the names of the markers on the *right*. The newly detected LGs were named N1 to N6. Distances (cM) between markers are shown to the *left*. *Underlined* markers are SSR markers (*asterisked* SSR markers showed significant segregation distortion at $P = 0.01$) developed in this study; others are AFLP markers

collection management and also for hybrid confirmation because selfing ratio of some *Zoysia* species (e.g., *Z. japonica*) was high and the true hybrid was difficult to distinguish based on morphology characters.

Mapping of SSR loci

Of the 1,003 polymorphic primer pairs, 170 (16.9%) amplified two bands in the parent (clone F02) of our mapping population. These 170 primer pairs detected 172 loci in the mapping population. Of these 172 mapped loci, 150 were mapped to the 24 existing AFLP-based linkage groups (LGs), 11 were mapped to six newly detected LGs (mostly small groups), and other 11 could not be mapped to any LGs. Among the 172 segregating loci, 10 showed segregating distortion at $P = 0.01$ and five were mapped in an interval of 21.7 cM on LG 3–19, which also included other distorted AFLP markers in this region.

Five pairs of existing AFLP-based LGs were joined into five LGs by adding the SSR data; e.g., AFLP LGs 1 and 25 were joined into LG 1–25 (Fig. 2). The SSR–AFLP-based linkage map consisted of 540 markers and covered a total map length of 1,187 cM, with an average spacing of 2.2 cM between markers. Mapped SSR markers were not spaced evenly throughout all LGs: in some LGs, only one or two markers were mapped (e.g., on LGs 4 and 12). Clusters of SSR markers in LGs were also found on LGs 1, 3–19, 5–9, and 6.

The ratio of polymorphic SSR markers in our mapping population, 16.9%, is very low; probably because our mapping population is an S_2 generation created by two generations of selfing of a single plant from only one clone, 'Tawarayama kita 1,' of *Z. japonica*, and *Z. japonica* has a higher selfing ratio than *Z. matrella* (Cai et al. 2004). However, if the mapping population is an F_2 population derived from the selfed progeny of other clones of *Z. matrella*, like Matsushima 5 or F08, the ratio of segregating loci can reach 30%, and an even

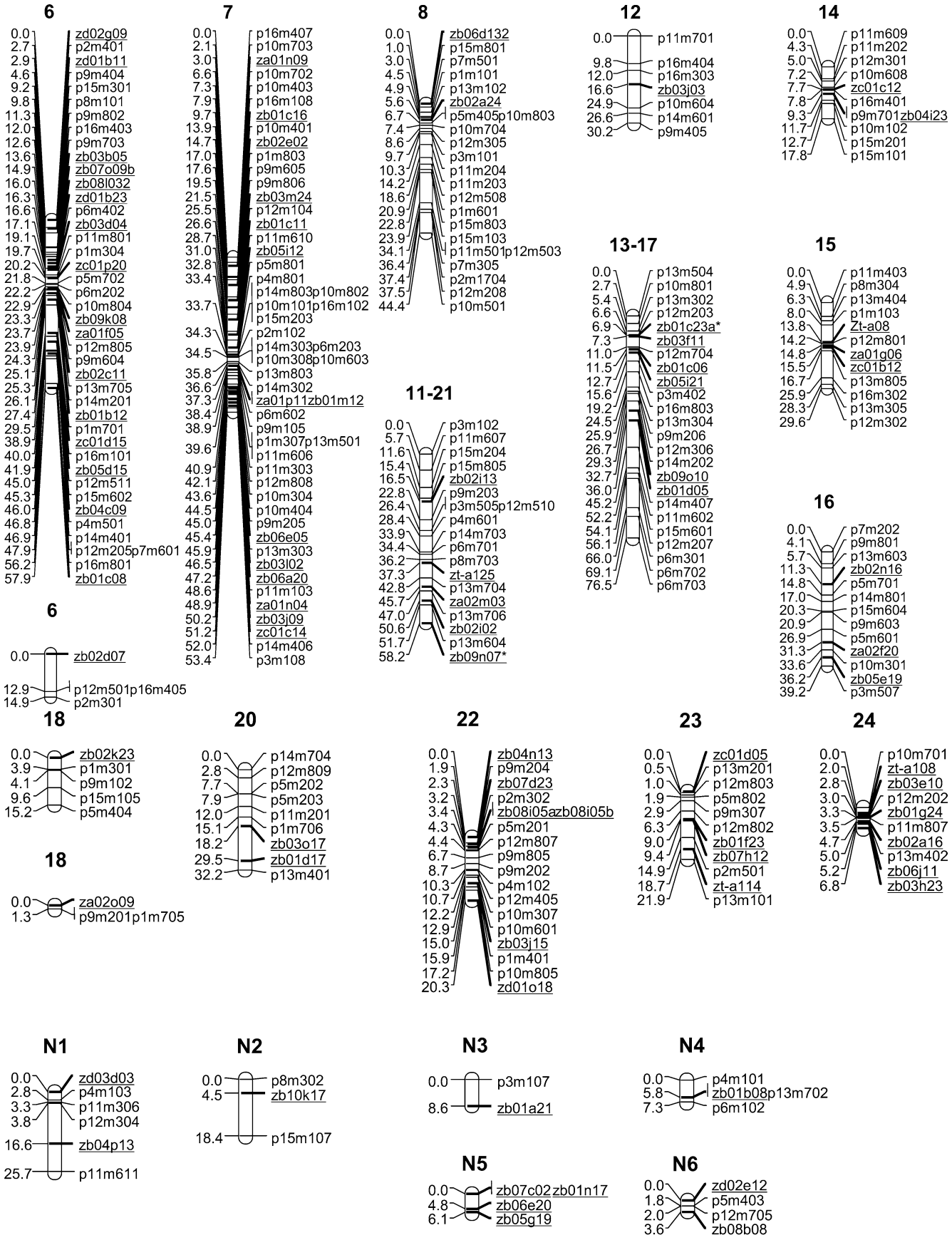
6
0.0 zd02g09
2.7 p2m401
2.9 zd01b11
4.6 p9m404
9.2 p15m301
9.8 p8m101
11.3 p9m802
12.0 p16m403
12.6 p9m703
13.6 zb03b05
14.9 zb07o09b
16.0 zb08l032
16.3 zd01b23
16.6 p6m402
17.1 zb03d04
19.1 p11m801
19.7 p1m304
20.2 zc01p20
21.8 p5m702
22.2 p6m202
22.9 p10m804
23.3 zb09k08
23.7 za01f05
23.9 p12m805
24.3 p9m604
25.1 zb02c11
25.3 p13m705
26.1 p14m201
27.4 zb01b12
29.5 p1m701
38.9 zc01d15
40.0 p16m101
41.9 zb05d15
45.0 p12m511
45.3 p15m602
46.0 zb04c09
46.8 p4m501
46.9 p14m401
47.9 p12m205p7m601
56.2 p16m801
57.9 zb01c08
7
0.0 p16m407
2.1 p10m703
3.0 za01n09
6.6 p10m702
7.3 p10m403
7.9 p16m108
9.7 zb01c16
13.9 p10m401
14.7 zb02e02
17.0 p1m803
17.6 p9m605
19.5 p9m806
21.5 zb03m24
25.5 p12m104
26.6 zb01c11
28.7 p11m610
31.0 zb05i12
32.8 p5m801
33.4 p4m801
33.7 p14m803p10m802
p10m101p16m102
p15m203
34.3 p2m102
34.5 p14m303p6m203
p10m308p10m603
35.8 p13m803
36.6 p14m302
37.3 za01p11zb01m12
38.4 p6m602
38.9 p9m105
39.6 p1m307p13m501
p11m606
40.9 p11m303
42.1 p12m808
43.6 p10m304
44.5 p10m404
45.0 p9m205
45.4 zb06e05
45.9 p13m303
46.5 zb03l02
47.2 zb06a20
48.6 p11m103
48.9 za01n04
50.2 zb03j09
51.2 zc01c14
52.0 p14m406
53.4 p3m108
8
0.0 zb06d132
1.0 p15m801
3.0 p7m501
4.5 p1m101
4.9 p13m102
5.6 zb02a24
6.7 p5m405p10m803
7.4 p10m704
8.6 p12m305
9.7 p3m101
10.3 p11m204
14.2 p11m203
18.6 p12m508
20.9 p1m601
22.8 p15m803
23.9 p15m103
34.1 p11m501p12m503
36.4 p7m305
37.4 p2m1704
37.5 p12m208
44.4 p10m501
12
0.0 p11m701
9.8 p16m404
12.0 p16m303
16.6 zb03j03
24.9 p10m604
26.6 p14m601
30.2 p9m405
14
0.0 p11m609
4.3 p11m202
5.0 p12m301
7.2 p10m608
7.7 zc01c12
7.8 p16m401
9.3 p9m701zb04i23
11.7 p10m102
12.7 p15m201
17.8 p15m101
13-17
0.0 p13m504
2.7 p10m801
5.4 p13m302
6.6 p12m203
6.9 zb01c23a*
7.3 zb03f11
11.0 p12m704
11.5 zb01c06
12.7 zb05i21
15.6 p3m402
19.2 p16m803
24.5 p13m304
25.9 p9m206
26.7 p12m306
29.3 p14m202
32.7 zb09o10
36.0 zb01d05
45.2 p14m407
52.2 p11m602
54.1 p15m601
56.1 p12m207
66.0 p6m301
69.1 p6m702
76.5 p6m703
15
0.0 p11m403
4.9 p8m304
6.3 p13m404
8.0 p1m103
13.8 Zt-a08
14.2 p12m801
14.8 za01g06
15.5 zc01b12
16.7 p13m805
25.9 p16m302
28.3 p13m305
29.6 p12m302
11-21
0.0 p3m102
5.7 p11m607
11.6 p15m204
15.4 p15m805
16.5 zb02i13
22.8 p9m203
26.4 p3m505p12m510
28.4 p4m601
33.9 p14m703
34.4 p6m701
36.2 p8m703
37.3 zt-a125
42.8 p13m704
45.7 za02m03
47.0 p13m706
50.6 zb02i02
51.7 p13m604
58.2 zb09n07*
16
0.0 p7m202
4.1 p9m801
5.7 p13m603
11.3 zb02n16
14.8 p5m701
17.0 p14m801
20.3 p15m604
20.9 p9m603
26.9 p5m601
31.3 za02f20
33.6 p10m301
36.2 zb05e19
39.2 p3m507
6
0.0 zb02d07
12.9 p12m501p16m405
14.9 p2m301
18
0.0 zb02k23
3.9 p1m301
4.1 p9m102
9.6 p15m105
15.2 p5m404
18
0.0 za02o09
1.3 p9m201p1m705
20
0.0 p14m704
2.8 p12m809
7.7 p5m202
7.9 p5m203
12.0 p11m201
15.1 p1m706
18.2 zb03o17
29.5 zb01d17
32.2 p13m401
22
0.0 zb04n13
1.9 p9m204
2.3 zb07d23
3.2 p2m302
3.4 zb08i05azb08i05b
4.3 p5m201
4.4 p12m807
6.7 p9m805
8.7 p9m202
10.3 p4m102
10.7 p12m405
12.2 p10m307
12.9 p10m601
15.0 zb03j15
15.9 p1m401
17.2 p10m805
20.3 zd01o18
23
0.0 zc01d05
0.5 p13m201
1.0 p12m803
1.9 p5m802
2.9 p9m307
6.3 p12m802
9.0 zb01f23
9.4 zb07h12
14.9 p2m501
18.7 zt-a114
21.9 p13m101
24
0.0 p10m701
2.0 zt-a108
2.8 zb03e10
3.0 p12m202
3.3 zb01g24
3.5 p11m807
4.7 zb02a16
5.0 p13m402
5.2 zb06j11
6.8 zb03h23
N1
0.0 zd03d03
2.8 p4m103
3.3 p11m306
3.8 p12m304
16.6 zb04p13
25.7 p11m611
N2
0.0 p8m302
4.5 zb10k17
18.4 p15m107
N3
0.0 p3m107
8.6 zb01a21
N4
0.0 p4m101
5.8 zb01b08p13m702
7.3 p6m102
N5
0.0 zb07c02zb01n17
4.8 zb06e20
6.1 zb05g19
N6
0.0 zd02e12
1.8 p5m403
2.0 p12m705
3.6 zb08b08

Fig. 2 (Contd.)

higher ratio (> 50%) can be expected in two-way pseudo-testcross F_1 populations (our unpublished data). In fact, the F_2 population derived from the selfed Matsushima 5 was under-construction.

Genomic constitution of *Zoysia* species

In autopolyploid species, for example, hexaploid timothy, most SSR markers could amplify up to six alleles in one sample alone (Cai et al. 2003). In contrast, among our 1,003 polymorphic SSR markers, only one primer pair (*ZA01B19*) could amplify four bands with the same sensitivity of the signal, but this marker was mapped as a single locus in our mapping population. We found two other loci (*ZB06D13* and *ZB08L03*) that could detect two loci each, and both loci from the same SSR primer pairs were mapped to different LGs; but the sensitivities of the bands were not same and one locus was much weaker than the other. As we reported earlier(Cai et al. 2004), we were unable to find any double duplex AFLP markers segregating 35:1, which is the ratio usually found in the F_2 progeny of autotetraploids within the same mapping population. The above results suggest that there is no evidence showing that the *Zoysia* species are autotetraploids.

Working with an interspecific mapping population, Yaneshita et al. (1999) reported that five pairs of LGs shared a series of duplicated loci with approximately the same order in several *Zoysia* species, these results mean that there are some homologous chromosome pairs present in the *Zoysia* species. In most typical allopolyploids with homologous chromosomes, such as wheat, oats, and tall fescue, most SSR primer pairs can amplify more than one locus, and the loci amplified by the same SSR primer are mapped on homologous chromosomes (Röder et al. 1998; Li et al. 2000; Saha et al. 2004, 2005, and our unpublished data). However, as mentioned above, we could not detect duplicate loci using the SSR markers developed in this study. The results suggested that the *Zoysia* species may inherit mostly like diploid, rather than tetraploids with homologous chromosomes. Since detecting duplicate loci using PCR-based markers like SSR and AFLP will need a higher level of sequence homologues than using RFLP markers, this may be one of the reasons why we could not find any duplicate loci in the *Zoysia* genome, using SSR markers.

We have, thus, reported the isolation and characterization of a large number of SSR sequences and the development of 1,044 SSR markers for zoysiagrass, and mapped 161 markers on the *Z. japonica* mapping population. The most of 1,044 SSR markers could also amplify SSR products in other *Zoysia* species, including *Z. tenuifolia* and *Zoysia macrantha* (our unpublished data). Thus, these SSR markers will provide an ideal marker system to assist with gene targeting, QTL mapping, variety or species identification, and marker-assisted selection in the *Zoysia* species. The primer information for other unmapped *Zoysia* SSR markers is available via material transfer agreements for research purposes or via license for commercial purposes.

Acknowledgments We thank the two anonymous reviewers for their valuable comments on this manuscript. This study was financially supported by a grant from the Japan Racing Association.

References

Akagi H, Yokozeki Y, Inagaki A, Fujimura T (1996) Microsatellite DNA markers for rice chromosomes. Theor Appl Genet 94: 61–67

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402

Arumuganathan K, Tallury SP, Fraser ML, Bruneau AH, Qu R (1999) Nuclear DNA content of thirteen turfgrass species by flow cytometry. Crop Sci 39:1518–1521

Barrett B, Griffiths A, Schreiber M, Ellison N, Mercer C, Bouton J, Ong B, Fordter J, Sawbridge T, Spangenberg G, et al (2004) A microsatellite map of white clover. Theor Appl Genet 109: 596–608

Bhattramakki D, Dong JM, Chhabra AK, Hart GE (2000) An integrated SSR and RFLP linkage map of *Sorghum bicolor* (L.) Moench. Genome 43:988–1002

Brent E, LaDeana H, Michael CW, Phil G (1998) Base-calling of automated sequencer traces using phred. I. Accuracy assessment. Genome Res 8:175–185

Cai HW, Yuyama N, Tamaki H, Yoshizawa A (2003) Development and characterization of simple sequence repeat (SSR) markers in hexaploid forage grass timothy (*Phleum pratense* L.). Theor Appl Genet 107:1337–1349

Cai HW, Inoue M, Yuyama N, Nakayama S (2004) An AFLP-based linkage map of zoysiagrass (*Zoysia japonica*). Plant Breed 123:543–548

Cardle L, Ramsay L, Milbourne D, Macaulay M, Marshall D, Waugh R (2000) Computational and experimental characterization of physically clustered simple sequence repeats in plants. Genetics 156:847–854

Chen X, Temnykh S, Xu Y, Cho YG, McCouch SR (1997) Development of a microsatellite framework map providing genome-wide coverage in rice (*Oryza sativa* L.). Theor Appl Genet 95:553–567

Chen X, Cho YG, McCouch SR (2002) Microsatellites in *Oryza* and other plant species. Mol Gen Genomics 268:331–343

Cordeiro GM, Taylor GO, Henry RJ (2000) Characterization of microsatellite markers from sugarcane (*Saccharum* sp.), a highly polyploid species. Plant Sci 155:161–168

Danin-Poleg Y, Reis N, Tzuri G, Katzir N (2001) Development and characterization of microsatellite markers in *Cucumis*. Theor Appl Genet 102:61–72

Ebina M, Kobayashi M, Kasuga S, Araya H, Nakagawa H (1999) An AFLP based genome map of zoysiagrass. In: Plant and animal genome VII conference, San Diego, CA, USA, 17–21 January

Fahima T, Röder MS, Wendehake K, Kirzhner VM, Nevo E (2002) Microsatellite polymorphism in natural populations of wild emmer wheat, *Triticum dicoccoides*, in Israel. Theor Appl Genet 104:17–29

Forbes I Jr (1952) Chromosome numbers and hybrids in *Zoysia*. Agron J 44:147–151

Fukuoka H (1989) Breeding of *Zoysia* spp (in Japanese). J Jpn Soc Turfgrass Sci 17:183–190

Gao LF, Tang JF, Li HW, Jia JZ (2003) Analysis of microsatellites in major crops assessed by computational and experimental approaches. Mol Breed 12:245–261

Hedrick PW (1985) Genetics of populations. Jones and Bartlett Publ Inc., Boston MA

Hirata M, Fujimori M, Komatsu T (2000) Development of simple sequence repeat (SSR) markers in Italian ryegrass. In: Proceedings of molecular breeding of forage crops 2000. Lorne and Hamilton, Victoria, Australia, 19–24 November, p 32

Huang XQ, Boener AB, Röder MS, Ganal MW (2002) Assessing genetic diversity of wheat (*Triticum aestivum* L.) germplasm using microsatellite markers. Theor Appl Genet 105:699–707

Jarret RL, Merrick LC, Holms T, Evans J, Aradhya MK (1997) Simple sequence repeats in watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai). Genome 40:433–441

Jones ES, Dupal MP, Kolliker R, Drayton MC, Forster JW (2001) Development and characterization of simple sequence repeat (SSR) markers for perennial ryegrass (*Lolium perenne* L.). Theor Appl Genet 102:405–415

Kantety RV, La Rota M, Matthews DE, Sorrells ME (2002) Data mining for simple sequence repeats in expressed sequence tags from barley, maize, rice sorghum and wheat. Plant Mol Biol 48:501–510

Kitamura F (1989) The climate of Japan and its surrounding areas and the distribution and classification of zoysiagrasses. Int Turfgrass Soc Res J 6:17–21

Kolliker R, Jones ES, Drayton MC, Dupal MP, Forster JW (2001) Development and characterization of simple sequence repeat (SSR) markers for white clover (*Trifolium repens* L.). Theor Appl Genet 102:416–424

Kong L, Dong J, Hart GE (2000) Characteristics, linkage-map positions, and allelic differentiation of *Sorghum bicolor* (L.) Moench DNA simple-sequence repeats (SSRs). Theor Appl Genet 101:438–448

Kosambi DD (1944) The estimation of map distance from recombination values. Ann Eugen 12:172–175

Li CD, Rossnagel BG, Scoles GJ (2000) The development of oat microsatellite markers and their use in identifying relationships among *Avena* species and oat cultivars. Theor Appl Genet 101:1259–1268

Litt M, Luty JA (1989) A hypervariable microsatellite revealed by in vitro amplification of a dinucleotide repeat within the cardiac muscle actin gene. Am J Hum Genet 44:397–401

Matsuoka Y, Mitchell SE, Kresovich S, Goodman M, Doebley J (2002) Microsatellites in *Zea*—variability, patterns of mutations, and use for evolutional studies. Theor Appl Genet 104:436–450

McCouch SR, Teytelman L, Xu YB, Lobos KB, Clare K, Walton M, Fu BY, Maghirang R, Li ZK, Xing YZ, et al (2002) Development and mapping of 2240 new SSR markers for rice (Oryza sativa L.). DNA Res 9:199–207

Murray MG, Thompson WF (1980) The isolation of high molecular weight plant DNA. Nucleic Acids Res 8:4321–4325

Panaud O, Chen X, McCouch SR (1996) Development of microsatellite markers and characterization of simple sequence length polymorphism (SSLP) in rice (*Oryza sativa* L.). Mol Gen Gent 252:597–607

Peng JH, Fahima T, Röder MS, Li YC, Dahan A, Grama A, Ronin YI, Korol AB, Nevo E (1999) Microsatellite tagging of the stripe-rust resistance gene *YrH52* derived from wild emmer wheat, *Triticum dicoccoides*, and suggestive negative crossover interference on chromosome 1B. Theor Appl Genet 98:862–872

Rampling LR, Harker N, Shariflou MR, Morell MK (2001) Detection and analysis systems for microsatellite markers in wheat. Aust J Agric Res 52:1131–1141

Ramsay L, Macaulay M, Ivanissevich SD, MacLean K, Cardle L, Fuller J, Edwards KJ, Tuvesson S, Morgante M, Massari A, et al (2000) A simple sequence repeat-based linkage map of barley. Genetics 156:1997–2005

Rossetto M, McLauchlan A, Harriss FCL, Henry RJ, Baverstock PR, Lee LS, Maguire TL, Edwards KJ (1999) Abundance and polymorphism of microsatellite markers in the tea tree (*Melaleuca alternifolia* Myrtaceae). Theor Appl Genet 98:1091–1098

Röder MS, Plaschke J, Konig SU, Borner A, Sorrells ME, Tanksley SD, Ganal MW (1995) Abundance, variability and chromosomal location of microsatellites in wheat. Mol Gen Genet 246:327–333

Röder MS, Korzun V, Wendehake K, Plaschke J, Tixier MH (1998) A microsatellite map of wheat. Genetics 149:2007–2023

Saal B, Wricke G (1999) Development of simple sequence repeats in rye (*Secale cereale* L.). Genome 42:964–972

Saha MC, Rouf Mian MA, Eujayl I, Zwonitzer JC, Wang L, May GD (2004) Tall fescue EST-SSR markers with transferability across several grass species. Theor Appl Genet 109:783–791

Saha MC, Mian R, Zwonitzer JC, Chekhovskiy K, Hopkins AA (2005) An SSR- and AFLP-based genetic linkage map of tall fescue (*Festuca arundinacea* Schreb.). Theor Appl Genet 110:323–336

Schloss SJ, Mitchell SE, White GM, Kukatla R, Bowers JE, Paterson AH, Kresovich S (2002) Characterization of RFLP probe sequence for gene discovery and SSR development in *Sorghum bicolor* (L.) Moench. Theor Appl Genet 105:912–920

Sharopova N, McMullen MD, Schultz L, Schroeder S, Sanchez-Villeda H, Gardiner J, Bergstrom D, Houchins K, Melia-Hancock S, Musket T, et al (2002) Development and mapping of SSR markers for maize. Plant Mol Biol 48:463–481

Shoji S (1983) Species ecology of zoysiagrass (in Japanese). J Jpn Soc Turfgrass Sci 12:105–110

Stam P (1993) Construction of integrated genetic linkage maps by means of a new computer package, JoinMap. Plant J 3:739–744

Tang S, Yu JK, Slabaugh MB, Shintani DK, Knapp SJ (2002) Simple sequence repeat map of the sunflower genome. Theor Appl Genet 105:1124–1130

Taramino G, Tingey S (1996) Simple sequence repeats for germplasm analysis and mapping in maize. Genome 39:277–287

Tautz D, Trick M, Dover GA (1986) Cryptic simplicity in DNA is a major source of genetic variation. Nature 322:652–656

Temnykh S, Park WD, Ayers N, Cartinhour S, Hauck N, Lipovich L, Cho YG, Ishii T, McCouch SR (2000) Mapping and genome organization of microsatellite sequences in rice (*Oryza sativa* L.). Theor Appl Genet 100:697–712

Temnykh S, DeClerck G, Lukashova A, Lipovich L, Cartinhour S, McCouch SR (2001) Computational and experimental analysis of microsatellites in rice (*Oryza sativa* L.): frequency, length variation, transposon association, and genetic marker potential. Genome Res 11:1441–1452

Tsuruta S, Ebina M, Kimura T, Yamamoto T, Hashiguchi M, Matsuo T, Akashi R, Takahara M, Kobayashi M, Nakagawa H (2003) Development of simple sequence repeat (SSR) markers in zoysiagrass. In: Plant and animal genomes XI conference, San Diego, CA, USA, 11–15 January

Voorrips RE (2002) MapChart, software for the graphical presentation of linkage maps and QTLs. J Hered 93:77–78

Wang Z, Weber JL, Zhong G, Tanksley SD (1994) Survey of plant short tandem DNA repeats. Theor Appl Genet 88:1–6

Wu KS, Tanksley SD (1993) Abundance, polymorphism and genetic mapping of microsatellites in rice. Mol Gen Genet 241:225–235

Xie CJ, Sun QX, NI ZF, Yang TM, Nevo E, Fahima T (2002) Chromosomal location of a *Triticum dicoccoides*-derived powdery mildew resistance gene in common wheat by using microsatellite markers. Theor Appl Genet 104:1164–1172

Yaneshita M, Kaneko S, Sasakuma T (1999) Allotetraploidy of *Zoysia* species with 2n = 40 based on RFLP genetic map. Theor Appl Genet 98:751–756